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Effect of Mono-oxygenase Inhibitors on Uptake, Metabolism and Phytotoxicity of Propanil in Resistant Biotypes of Jungle-Rice, *Echinochloa Colona*

Jon M. Leah,[a]* John C. Caseley,[a] Charles R. Riches[b] & Bernal E. Valverde[c]

[a] Department of Agricultural Sciences, University of Bristol, Institute of Arable Crops Research, Long Ashton Research Station, Bristol BS18 9AF, UK
[b] Natural Resources Institute, Chatham Maritime, Kent, ME4 4TB, UK
[c] Area de Fitoproteccion, CATIE, Turrialba 7170, Costa Rica

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Abstract: The effect of the mono-oxygenase inhibitors tridiphane, piperonyl butoxide and prochloraz on propanil uptake, metabolism and phytotoxicity was measured in a resistant (R) biotype of *Echinochloa colona*. The uptake of propanil was not significantly affected by any of the three mono-oxygenase inhibitors. The first metabolite of propanil metabolism, 3,4-dichloroaniline, was found to accumulate to higher levels in *E. colona* treated with each of the mono-oxygenase inhibitors mixed with formulated propanil, compared to propanil applied alone.

Accumulation of further metabolites of propanil (glucosyl-3,4-dichloroaniline and bound products) was reduced in the presence of mono-oxygenase inhibitors, compared with propanil application alone. Leaf damage caused by a single drop of propanil compared to propanil + mono-oxygenase inhibitor was used to assess the degree of propanil tolerance in *E. colona* biotypes. Leaf damage was significantly greater in propanil + mono-oxygenase inhibitor treatments. No leaf damage was observed in mono-oxygenase inhibitor treatments alone at the concentrations used.

Peroxidase activity was measured in crude extracts of the R-biotype of *E. colona* using 3,4-dichloroaniline as substrate, in the presence and absence of mono-oxygenase inhibitors and the specific peroxidase inhibitor salicylhydroxamic acid. Peroxidase activity was inhibited by all three mono-oxygenase inhibitors at 10 μM and by salicylhydroxamic acid at 1 μM. Glucosyl-3,4-dichloroaniline was found not to be a substrate for peroxidase activity.

These results suggest that the incorporation of 3,4-dichloroaniline into bound residues involves peroxidase activity which can be inhibited by mono-oxygenase inhibitors. When peroxidase activity is inhibited, the precursor metabolite 3,4-dichloroaniline accumulates, and propanil resistance in *E.* colona is reduced, possibly as a consequence of phytotoxicity of this metabolite and/or product inhibition of the first step in propanil metabolism, responsible for the formation of 3,4-dichloroaniline. Glasshouse trials have demonstrated that the application of mono-oxygenase inhibitors, (particularly tridiphane which is also known to inhibit glutathione transferase activity) with propanil offers a promising approach to the control of propanil resistant biotypes of Jungle-Rice.

Key words: propanil, Echinochloa, resistance, mono-oxygenase, metabolism, uptake

* To whom correspondence should be addressed at: AgrEvo UK Ltd, Toxicokinetics, Chesterford Park, Saffron Walden, Essex, CB10 1XL, UK.

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1 INTRODUCTION

The post-emergence herbicide, propanil (3,4-dichloropropionanilide) is important for control of grass weeds such as *Echinochloa* sp. in rice crops.[1,2] The basis of selectivity between propanil-susceptible biotypes of *Echinochloa colona* (L.) Link and resistant rice has been established as the differential metabolism of this herbicide[3,4] whereby propanil is firstly converted to 3,4-dichloroaniline *via* a reaction catalysed by an aryl acylamidase.[5,6] Further metabolism of propanil is thought to involve glucosylation[7,8] followed by incorporation into lignin[9] and/or pectin cell wall polymers.[10]

Extensive use of propanil has resulted in the selection of resistant biotypes of *E. colona*[11] which has become a major problem weed in rice crops. Attempts to improve efficacy of propanil have included the use of carbamate and organophosphorus insecticides as synergists[12–14] since these compounds are known to inhibit aryl acylamidase activity[15] thereby enhancing the phytotoxicity of propanil by reducing its metabolism.[3,4,16,17]

However, tolerance to propanil in *E. colona* increases with plant age, possibly due to an observed decrease in uptake of this herbicide.[18] Consequently, selective phytotoxicity of propanil based upon differences in aryl acylamidase-mediated metabolism is progressively reduced with plant age, suggesting that even propanil–insecticide mixtures would have limited use in the control of older *E. colona* plants.

Whilst improved formulation may increase uptake of propanil,[19] another approach to improving propanil action may be to inhibit further metabolism prior to incorporation of 3,4-dichloroaniline into plant structural polymers. Enzymes that are important for lignin biosynthesis (cinnamic acid-4-hydroxylase) and polymerisation (peroxidases)[20] may be ideal targets for mono-oxygenase inhibitors which could synergise propanil. Reduced incorporation of 3,4-dichloroaniline into plant polymers would be expected to result in the accumulation of this metabolite, which exhibits some degree of phytotoxicity and, unusually in xenobiotic metabolism, is less polar than the parent molecule, propanil.[18] Propanil metabolism could then be reduced by product inhibition of the aryl acylamidase reaction, resulting in improved phytotoxicity.

Field trials using propanil mixed with the glutathione transferase[21] and mono-oxygenase[22] inhibitor herbicide, tridiphane, have demonstrated that *E. crus-galli* can be effectively controlled in rice[23] with no significant increase in injury to the crop. Interactions of propanil and mono-oxygenase inhibitors (tridiphane, piperonyl butoxide[22] and the triazole, prochloraz[24]), were therefore investigated in the present study.

The aims of this study were to (1) demonstrate altered metabolism of propanil in the presence of the following mono-oxygenase inhibitors: the herbicide, tridiphane, the azole fungicide, prochloraz and the pyrethrin insecticide synergist, piperonyl butoxide, (2) investigate whether propanil phytotoxicity is increased in the presence of mono-oxygenase inhibitors and (3) determine whether peroxidation of 3,4-dichloroaniline and glucosyl-dichloroaniline is inhibited by mono-oxygenase inhibitors.

2 MATERIALS AND METHODS

2.1 Plant material and growth conditions

Biotypes of *E. colona* were collected from rice fields in Colombia and designated propanil-susceptible (S) and resistant (R) after assessment by spray studies.[11] In the uptake and metabolism studies, a biotype which was resistant to 16 kg AI ha^{-1} was used as a representative of a resistant population.[6] *E. colona* seeds were pregerminated in Petri dishes for four days prior to planting in a sandy loam soil containing 300 mg g^{-1} coarse grit. Plants were grown at 25°C, 50% relative humidity and 14-h photoperiod in a controlled-environment growth cabinet. Unless otherwise stated, all treatments were initiated 7 h through the photoperiod. Plants were watered from above twice daily. All studies were carried out on recently fully expanded or younger leaves. Only treated leaves were used for uptake and metabolism studies since translocation of propanil outside the leaf was assumed to be negligible based on previous experiments.

For whole-plant studies, the *E. colona* biotype used came from a rice field in Costa Rica on which propanil had been used for more than 20 years. Plants were grown, three to a 9-cm pot, in a glasshouse with a 16-h photoperiod at 30°C and at 28°C during darkness. Relative humidity was maintained at 85($\pm$10)%. Plants were watered from above twice daily.

2.2 Uptake and metabolism of propanil

A 3-μl drop of [*phenyl-U*-^{14}C]propanil (0·2 μCi) formulated in a commercial 360 g $litre^{-1}$ EC ('Stam' F-34, Rohm and Haas) at a final concentration of 32 mM propanil was applied using a micro-applicator (PAX-100, Burkard Scientific) to the midrib of the adaxial leaf surface of *E. colona* in the presence of 10 μM tridiphane (diluted from a 480 g $litre^{-1}$ EC, 'Nelpon'; Dow, with 20% (v/v) methanol), 10 μM prochloraz (diluted from a 400 g $litre^{-1}$ EC, 'Sportak'; AgrEvo with 20% (v/v) methanol) and piperonyl butoxide (unformulated technical grade, diluted in 20% (v/v) methanol). In all cases, the last fully expanded leaf was treated. Leaves were harvested at set time intervals after application and washed in absolute methanol (3 × 3·33 ml). Washings from each growth stage were pooled and an aliquot

taken to assess uptake (amount of [^{14}C]propanil absorbed by the leaf) by liquid scintillation spectrometry using HiSafe III as scintillant. Washed leaves were homogenised in absolute methanol (3·33 ml) using a glass homogeniser. This extract was centrifuged at 7500*g* for 3 min and the pellet re-extracted twice in the same volume of methanol to produce 10 ml total volume. A 1·0-ml aliquot was removed and counted for ^{14}C activity to confirm uptake measurements. The remaining 9 ml of extract was evaporated to dryness under vacuum and then re-dissolved in the minimum volume of solvent mixture used for TLC separation. This concentrate was applied to a 20 × 20 cm glass-backed silica TLC plate (Kieselgel 60, F254, Merck) and components separated using chloroform + methanol + pyridine (100 + 5 + 1 by volume) as solvent system. Plates were dried and ^{14}C-labelled components detected and quantified using a Rita 68000 radio-TLC plate scanner. Components were identified by co-migration with known standards as follows: propanil, 3,4-dichloroaniline and glucosyl-3,4-dichloroaniline all of which fluoresce under UV light. R_f values for components using this separation system were as follows: propanil 0·5, 3,4-dichloroaniline 0·56 and glucosyl-3,4-dichloroaniline 0·017. The glucosyl conjugate was synthesised as described previously.[7] Glucose monohydrate (2·5 g, 12·6 mmol) and 3,4-dichloroaniline (2·2 g, 13·8 mmol) were dissolved in absolute methanol (HPLC grade; 15 ml) containing acetic acid (0·25 ml). This mixture was refluxed for 30 min and then left to cool at 4°C. The precipitate formed was recrystallised from methanol and this extract used as a standard.

2.3 Assessment of propanil resistance

Three-microlitre drops of propanil 400 g litre^{-1} EC ('Stam' F-34) equivalent to the label recommended field dose of 1·4 kg AI ha^{-1} in 200 litre spray volume (32 mM propanil) were applied to the midrib on the adaxial leaf surface of *E. colona* plants (one drop per leaf). In all cases, the last fully expanded leaf was treated. After four days, the lengths of necrotic lesions which formed toward the leaf tip in the direction of the transpiration stream were measured. Lesion length was found to be a good indicator of the degree of propanil resistance compared to results from propanil spray studies in *E. colona*. Lesion length was assessed at the 15-day (four-leaf) and 35-day-old growth stages of *E. colona*. Similar experiments were performed to assess the effect upon propanil phytotoxicity as a result of simultaneous treatment with tridiphane EC ('Neplon'), prochloraz EC ('Sportak') or piperonyl butoxide, each diluted in 20% (v/v) methanol and added to propanil EC to a final concentration of 10 μM.

2.4 Measurement of peroxidase activity

Crude extracts of 15-day-old and 35-day-old *E. colona* were made from 15 individual plants for each essay ($n = 3$) using a pre-cooled mortar and pestle in citrate-phosphate buffer (100 mM; pH 4·0) to produce a 200 mg fresh weight ml^{-1} homogenate. This extract was filtered through two layers of cheesecloth and centrifuged at 13 500*g* for 15 min. The supernatant was assayed immediately.

Peroxidase activity was measured spectrophotometrically using the method described by Laurent[25] with modifications. The substrate 3,4-dichloroaniline and metabolite, glucosyl-3,4-dichloroaniline were dissolved in 20% (v/v) methanol. The assay incubation mixture contained 400 μl of crude extract, 60 μl of 3,4-dichloroaniline (50 mM), 30 μl of freshly prepared hydrogen peroxide (5 mM) and 30 μl of 60 μM inhibitor (final concentration of 10 μM) made up to a total volume of 600 μl with citrate-phosphate buffer (50 mM; pH 4·0). The reaction was started by addition of crude extract at 20°C. Absorbance was monitored over a period of 2 min at 455 nm. Inhibitors were dissolved and diluted in 20% (v/v) methanol. Control assays were performed where inhibitor was replaced with the same volume of methanol.

2.5 Whole plant studies

Propanil alone or as tank mixture with tridiphane, prochloraz or piperonyl butoxide was applied to 15-day-old *E. colona* plants with three to four leaves using a laboratory track sprayer calibrated to deliver 200 litre ha^{-1}. The chemical products used for uptake and metabolism studies were also applied to whole plants. Tridiphane, prochloraz and piperonyl butoxide were evaluated in separate experiments; fresh weight of foliage from treated and untreated control plants was assessed at 14 days after application respectively of tridiphane or prochloraz and 30 days in the case of piperonyl butoxide. After spraying, pots were arranged in randomised complete blocks with three replicates of the treatments shown in Table 2.

3 RESULTS

Uptake of [^{14}C]propanil was not significantly altered by the presence of tridiphane, prochloraz or piperonyl butoxide (Fig. 1). Approximately 80% of the applied ^{14}C activity was absorbed by leaves of *E. colona* three days after treatment.

Metabolism of propanil resulted in the rapid accumulation of 3,4-dichloroaniline which reached a plateau after 24 h at approximately 40% of absorbed ^{14}C activity and remained at this level throughout the six-day

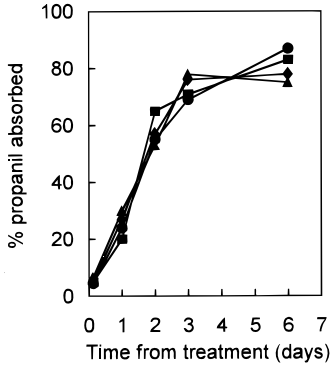

Fig. 1. Uptake of propanil in an R-biotype of *Echinochloa colona* at the four-leaf growth stage (15 days old) (●) alone, and in presence of (▲) tridiphane, (■) prochloraz and (◆) piperonyl butoxide at 10 μM. Uptake is expressed as percentage of ^{14}C activity applied to the leaf. Results are means ($n = 3$) where standard errors are < 5% of means in all cases.

period of study (Fig. 2A). Further metabolites accumulated to about 40% of absorbed ^{14}C activity over the six-day period while propanil levels decreased to about 30%. The presence of tridiphane (Fig. 2B) resulted in the rapid accumulation of 3,4-dichloroaniline within 24 h which reached a plateau at an increased level of about 55% of absorbed ^{14}C activity, with propanil levels reduced to about 40%. The accumulation of

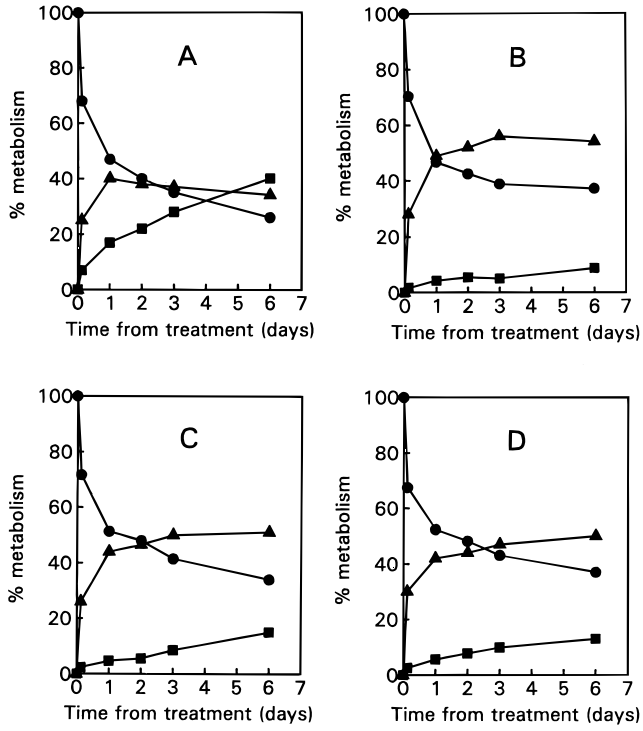

Fig. 2. Metabolism of propanil in *Echinochloa colona* (R-biotype) at the four-leaf growth stage (15 days old) (A) without inhibitor, (B) with tridiphane, (C) with prochloraz and (D) with piperonyl butoxide, each at 10 μM. Metabolism is expressed as percentage of ^{14}C activity absorbed onto the leaf. Results are means ($n = 3$) where standard errors are < 5% of means in all cases. Metabolites are as follows: (●) Propanil; (▲) 3,4-dichloroaniline; (■) further metabolites.

TABLE 1
Effect of Cytochrome P450 Inhibitors upon Propanil Phytotoxicity in Propanil-Resistant *Echinochloa colona* in 15- and 35-Day-Old Plants

	Lesion length[a] *(mm)*	
Treatment	*15-day*	*35-day*
Propanil alone	7·6	5·3
Propanil + tridiphane	25·2	20·8
Propanil + prochloraz	18·7	15·6
Propanil + piperonyl butoxide	15·5	10·4

[a] Values are expressed as means ($n = 10$) four days after leaf drop application where standard errors are < 5% of means.

further metabolites were markedly reduced compared to propanil treatment alone, reaching approximately 10% of absorbed ^{14}C activity compared to 40%. Similarly altered patterns of propanil metabolism were found in the presence of prochloraz (Fig. 2C) and piperonyl butoxide (Fig. 2D).

Phytotoxicity due to propanil, as assessed by measurement of the area of leaf necrosis (lesion length) formed around a single drop, was found to be greatly enhanced in the presence of tridiphane and, to a lesser

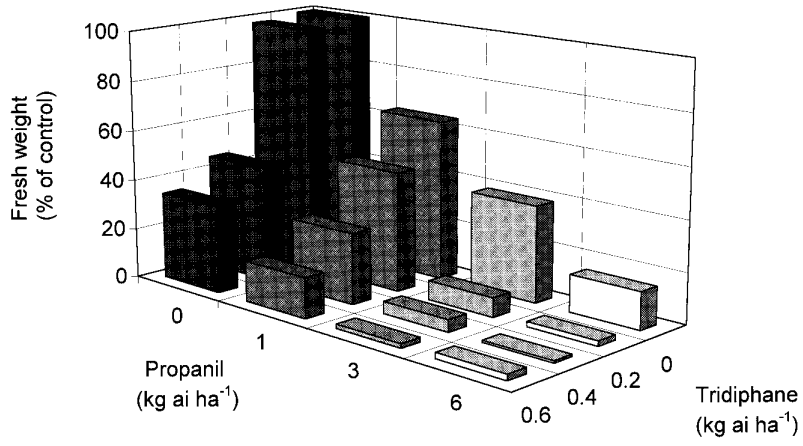

Fig. 3. Enhancement of propanil growth inhibition of *Echinochloa colona* by tridiphane at the three- to four-leaf growth stage. Fresh weight as percentage of untreated plants at 14 days following spraying. Mean fresh weight untreated plants = 4·5(± 0·2) g .

TABLE 2
Inhibition of 3,4-Dichloroaniline Peroxidase Activity in *Echinochloa colona* (R) in 15- and 35-Day-Old Plants

Inhibitor	*Concentration* (μM)	*Relative % activity* (%)	
		15 days	*35 days*
Control	—	100[a]	100[b]
Tridiphane	10	34	37
Prochloraz	10	62	65
Piperonyl butoxide	10	72	76
Salicylhydroxamic acid	1	59	62

[a] Maximum peroxidase activity equivalent to an absorbance change of 0·485 min^{-1} at pH 4·0 and 25°C.
[b] Maximum peroxidase activity equivalent to an absorbance change of 0·675 min^{-1} at pH 4·0 and 25°C.
Activities are expressed as means ($n = 3$) where standard errors are $< 3\%$ of means.

extent, in the presence of prochloraz and piperonyl butoxide (Table 1). Younger plants (15 days old) were more susceptible to propanil damage, both in the presence and absence of mono-oxygenase inhibitors, than older plants (35 days old). Treatment of plants with mono-oxygenase inhibitors alone produced no leaf necrosis at the concentration used.

Peroxidase activity using 3,4-dichloroaniline as substrate was about 30% lower in 15-day- compared to 35-day-old *E. colona* (R) plants (Table 2). Tridiphane produced a 66% inhibition of peroxidase activity in 15-day-old plants at 10 μM which was only slightly less in 35-day-old plants. Prochloraz and piperonyl butoxide also inhibited peroxidase activity but were approximately 50% less effective at 10 μM compared to tridiphane. The specific peroxidase inhibitor, salicylhydroxamic acid was inhibitory at 1 μM and was used as a positive control.

Applied to 15-day-old *E. colona* seedlings, with three to four true leaves, tridiphane at 0·4 kg AI ha^{-1} reduced plant fresh weight by more than 50% compared to untreated plants (Fig. 3). Propanil-induced growth reduction was greatly enhanced by the simultaneous application of tridiphane.

Piperonyl butoxide and prochloraz, at rates up to 0·4 and 0·6 kg AI ha^{-1} respectively did not inhibit the growth of *E. colona* seedlings (Table 3). Propanil activity was enhanced when applied at 3·0 kg AI ha^{-1} in combination with 0·2 kg AI ha^{-1} piperonyl butoxide or when applied at 1·0 kg AI ha^{-1} in combination with 0·6 kg AI ha^{-1} prochloraz.

4 DISCUSSION

The increased accumulation of the first metabolite of propanil, 3,4-dichloroaniline, in the presence of mono-

TABLE 3
Effect of Application of Propanil Alone and in Combination with P450 Inhibitors to 15-Day-Old Propanil-Resistant *Echinochloa colona* Seedlings

		Fresh weight (% *of untreated*) ($\pm SE$)			
		Propanil (*kg AI* ha^{-1})			
		0	*1·0*	*1·5*	*3·0*
Prochloraz[a] (kg AI ha^{-1})	0	100[b]	61·1 (±9·3)	—[d]	38·5 (±14·3)
	0·4	116·0 (±13·06)	53·1 (±7·5)	—	32·8 (±10·0)
	0·6	110·3 (±1·3)	33·0 (±6·5)	—	25·0 (±9·2)
Piperonyl butoxide[a] (kg AI ha^{-1})	0	100[c]	—	79·6 (±1·1)	60·2 (±3·8)
	0·2	95·4 (±2·9)	—	81·3 (±7·0)	34.0 (±19·4)
	0·4	100·1 (±3·4)	—	78·6 (±2·6)	34·4 (±15·9)

[a] Measurements 14 days after treatment for prochloraz and 30 days for piperonyl butoxide.
[b] Mean fresh weight untreated plants = 3·2 ± 0·2 g.
[c] Mean fresh weight untreated plants = 6·8 ± 0·4 g.
[d] Combination not tested.

oxygenase inhibitors, and the marked decrease in production of further metabolites demonstrates that the incorporation of 3,4-dichloroaniline is being inhibited. Whilst such incorporation reactions may involve both mono-oxygenase-dependent hydroxylation and peroxidation, it is the latter reaction that is more likely to be important for polymerisation of 3,4-dichloroaniline into lignin and associated cell wall polymers. Consequently, the inhibition of peroxidase activity by mono-oxygenase inhibitors may be important in reducing incorporation of 3,4-dichloroaniline. Since peroxidase activity was measured in crude extracts in the absence of protectants, it is likely that such activity is independent of mono-oxygenase systems, which are notoriously unstable.

In the case of tridiphane, there may be an additional inhibitory interaction with propanil metabolism, since this compound has been shown to act as both a mono-oxygenase[22] and a glutathione transferase[21] inhibitor.

Glucosylated 3,4-dichloroaniline was not a substrate for peroxidase activity in *E. colona*, suggesting that the glucosylation reaction may be reversible and that 3,4-dichloroaniline is directly incorporated into polymers as previously proposed.[26] Indeed, there is evidence to suggest that glucosylation of chlorinated anilines is both spontaneous, reversible and pH-dependent,[27] although more rapid enzymic glucosylation has been reported in soybean.[28]

The reversible glucosylation of 3,4-dichloroaniline has the physiological advantage of considerably reducing translocation of this relatively non-polar metabolite by increasing its polarity and reducing its toxicity (Leah, J. M., unpublished). The instability of the glucosyl conjugate may therefore enable 3,4-dichloroaniline to be reformed immediately prior to incorporation into polymers. Furthermore, the measurement of 3,4-dichloroaniline in the present study may represent a significant amount of glucosylated metabolite which could break down during processing. This would account for the apparent accumulation of extracted 3,4-dichloroaniline in the absence of excessive toxicity which might be expected for such a non-polar molecule.

Application to *E. colona* seedlings indicated that the combination of mono-oxygenase inhibitors, particularly tridiphane, with propanil would appear to offer a promising approach to controlling resistant biotypes of *E. colona*, provided selective phytotoxicity in the crop is maintained. Such mixtures have previously been shown to improve the control of *E. crus-galli* in rice without excessive damage to the crop.[23] This work utilised technical grade piperonyl butoxide which had not been formulated for application to plants and formulations of tridiphane and prochloraz not previously tested in mixture with formulated propanil. Further field trials with doses and formulation with appropriate surfactant systems are needed to identify combinations which optimise control of *E. colona* in the field. Currently we are investigating three-way mixtures of propanil, amidase and mono-oxygenase inhibitors to combat resistance in *Echinochloa colona.*

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